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GENETIC ENGINEERING OF CLOSTRIDIUM DIFFICILE TOXIN A VACCINE

ANNUAL REPORT

Lycurgus L. Muldrow
Joe Johnson

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The major long range objective of this project is to use C. difficile toxin A as a model to add technical information in the area of vaccine biotechnology as it relates to recombinant DNA studies. However, prior to accomplishing this goal C. difficile toxin A DNA must be cloned, sequenced and characterized, which was the objective of the first year of work, and is reported in part in this document.

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SUMMARY

Recombinant DNA technology allows for an approach to subunit vaccine production that should provide advantages over existing techniques. Improvement of vaccine biotechnology in the area of recombinant DNA studies using Clostridium difficile toxin A as the model, is the major long range objective of this project. However, prior to accomplishing this goal C. difficile toxin genes must be cloned, sequenced and characterized, which was the objective of the first year of work, and is reported in part in this document. A genomic library in lambda gt11 of C. difficile chromosomal DNA was screened using anti-toxin A which resulted in the identification of one stable positive clone, lambda cd19. Verification of the immunological identity of the isolated toxin A gene fragment in lambda cd19 was determined by affinity purifying toxin A antibodies specific for lambda cd19 gene product, and using these selected antibodies to probe a Western blot of purified toxin A. The insert in lambda cd19 was demonstrated to be a 0.3 kb fragment by restriction digestion, and by hybridization of the clone to a chromosomal digest of C. difficile. The peptide coded for by the toxin A gene fragment in lambda cd19 was not cytotoxic for 3T3 mammalian tissue culture cells. Sequence analysis of DNA encoding for toxin A was determined using the Sanger chain-termination sequence procedure. The amino acid sequence was deduced for the DNA sequence and a hydropathy plot of the open reading frame given. Future studies within the next year will be centered around sequencing the entire toxin A gene and predicting epitopes.

FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

The investigator(s) have abided by the National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules (April 1982) and the Administration Practices Supplements.

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BACKGROUND

Recombinant DNA technology allows for an approach to subunit vaccine production that should provide advantages over existing techniques. Potential advantages of genetically engineered antigens for the preparation of vaccines are stability, purity, safety of preparation, price, lack of side effects and variety of serotypes. A number of determinants coding for antigens have been cloned from viruses, bacteria, parasites and toxins, with the ultimate goal of producing better vaccines and improving molecular techniques for the development of vaccines. Improvement of vaccine biotechnology in the area of recombinant DNA studies using Clostridium difficile toxin A as the model, is the major long range objective of this project. However, prior to accomplishing this goal C. difficile toxin genes must be cloned, sequenced and characterized, which was the objective of the first year of work, and is reported in part in this document.

1. Clostridium Difficile Toxin A Literature Review

C. difficile was first described in 1935 (1), but has only been recognized as a clinically significant pathogen within the last decade. This toxin producing strict anaerobic bacterium is the causative agent for the diarrheal syndrome termed antibiotic associated pseudomembranous colitis (PMC) (2,3,4,5). PMC is a disease of the lower gastrointestinal tract that can be histopathologically characterized by exudative plaques on the bowel mucosa. Pathogenicity and cytotoxicity associated with PMC has been linked to production of two toxins, A (enterotoxin) and B (cytotoxin). There have been a number of recent publications describing biological characteristics of toxins A and B (6,7,8,9,10,11.12). However the etiology of PMC does not only depend on colonization of C. difficile toxin producing strains, but other factors affecting the gastrointestinal tract may initiate the disease state, such as antibiotic therapy (13,14,15).

Evidence has developed which indicates that toxins A and B work synergistically to cause PMC (16,17). Lyerly and coworkers demonstrated that large doses of cytotoxin B have little effect when administered intragastrically to hamsters, unless this toxin is administered simultaneous to low non-toxic levels of enterotoxin A, or unless the gut has been subjected to mechanical injury (16). Rolfe and Kim demonstrated that immunization against toxoid B did not protect hamsters from clindamycin induced lethal ileocecitis, but toxoid A or a combination of toxoid A and B gave protection from lethal ileocecitis (17). These finding suggest that cytotoxin B is provided an opportunity to work, after enterotoxin A damages tissue.

Toxin A is a large protein which has been demonstrated to elicit a hemorrhagic fluid response in the rabbit intestinal loop assay, cause fluid accumulation in the suckling mouse assay (18,19), exhibit cytotoxic activity on mammalian tissue culture

cells (20), and bind to and agglutinate rabbit erythrocytes (21) The molecular weight of toxin A has been reported by several different groups using non-denaturing gels and gel filtration to be approximately 440,000 to 600,000 (22,23,24). However, elucidation of the quaternary structure of toxin A still requires additional work. Preliminary evidence by Rautenberg and Stender (25) using a 2-dimensional gel system, suggests that toxin A contains two large 230,000 dalton subunits and an unidentified number of smaller subunits of 35,000 dalton. These results do not contradict work done in other laboratories on SDS denaturing gels. Lyerly et al. demonstrated using monoclonal antibodies on Western blots of SDS gels the presence of an estimated 300,000 molecular weight band and numerous minor bands (26). Banno et al. also observed a large protein band on denaturing gels with a molecular weight of 190,000 to 200,000, however they did not observe minor bands (27).

Toxin A and toxin B are two different immunologically (27,28) and biochemically distinct proteins (30), however they share biochemical and immunological characteristics in common. The biochemical similarities that have been reported for these two toxins are: 1) they are heat labile at 56°C; 2) they are inactivated at pH 2; 3) they have a high degree of acidic and hydrophobic amino acids and low amounts of sulfur containing amino acids; and 4) they are susceptible to oxidation (22,28,29). Immunological evidence exist which show that toxins A and B share a common epitope. Three types of monoclonal antibodies against C. difficile's toxins have been observed, one type with epitopes for toxin A, one type with epitopes for toxin B, and another type with epitopes common to both toxins (26,30). These immunological results shows that toxin A and B have similar antigenic sites and that these proteins may share a common subunits.

Studies directed toward the molecular genetics or DNA of C. difficile have not been as numerous as those dealing with the pathology or biochemistry of toxins. DNA directed studies on this organism have included: 1) chromosomal location, homology, and transfer of tetracycline, erythromycin and clindamycin resistance determinants (31,32,33,34); 2) Survey and analysis of extrachromosomal elements (35,36); 3) chromosomal restriction endonuclease digestion for typing C. difficile (34,35); 4) cloning of a functional C. difficile promoter in E. coli (39); 5) cloning of C. difficile species-specific antigen in E. coli (40); 6) generation of a DNA hybridization probe for detection of C. difficile (41); and 7) cloning and analysis of toxin determinants (42,43,44,45,46,47,48,). It should be noted that only three of the ten references listed in sections 4 through 7 above on cloning (42,46,47) are in the form of publications, the other references were abstracts presented at the American Society for Microbiology annual meetings. None of these publications (42,46,47) report DNA sequence analysis of C. difficile genes; thus, molecular cloning and analysis of genes in this gram positive bacteria are still in the developmental stages.

2. Technical Objectives

A. Long Range Goals. The major long range objective of this project is to use C. difficile toxin A as a model to add technical information in the area of vaccine biotechnology as it relates to recombinant DNA studies. Recombinant DNA techniques will be used to identify and isolate specific C. difficile toxin A determinants which code for antigenic sites, with the ultimate goal of immunizing laboratory animals with these genetically engineered epitopes. These long range objectives will be accomplished by first sequencing the toxin gene and deducting the amino acid sequence. From the knowledge of the amino acid sequence, epitopes will be predicted. The predicted epitopes will be synthesized using a DNA synthesizer and cloned into the lacZ gene of the lambda gt11 expression vector. This cloning system will allow for rapid selection of specific antibodies that recognize cloned epitopes. Verification of cloned functional epitopes will be accomplished if affinity purified antibodies which specifically bind to the cloned epitopes, bind to a purified toxin A band during Western-blot analysis. A rapid tissue culture assay, utilizing 96-well microtiter plates, will be employed to determined if the affinity purified antibodies specific for the cloned epitopes can protect mammalian cells from the cytotoxic activity of the toxin. After the cloned epitopes have been characterized, the antigenic peptide-beta-galactosidase fusion proteins will be purified using a commercially available anti-beta-galactosidase column. These fusion proteins will be used to immunize mice. The animals will be challenged later with the purified toxin and the toxic bacterium in order to monitor the production of neutralizing antibodies. The above series of experiments represent a unique approach for prediction, identification and purification of antigenic sites, which may be used as a model for development of any subunit vaccine.

B. Immediate Goals. During the first year of this four year project, C. difficile toxin A gene fragments have been cloned, partially sequenced and characterized. During the next year the DNA sequence of this gene will be fully characterized, and epitopes will be predicted.

MATERIALS AND METHODS

1. Affinity Purification of Antibodies

Antibodies specific for the toxin-positive peptide encoded by recombinant lambda gt11 phage particles were affinity purified from toxin A antiserum using the procedure described by Lyon et al. (49) with the following variations. Recombinant phage were plated at a density of approximately 6,000 plaques per plate (150mm diameter). Nitrocellulose filters were overlaid on the agar plates and incubated at 37°C for 16 hours which allowed for binding of antigens. The filters were next removed and suspended overnight in a 1:50 dilution of E. coli adsorbed toxin

A antiserum. After washing the nitrocellulose paper twice in TTBS (0.05% tween 20, 50 mM tris, 150 mM NaCl, pH 8.0) and once in 10 ml saline, specific antibodies which bound to the nitrocellulose filters were eluted by adding 10 ml of glycine buffer per filter (0.2 M glycine, 0.15 M NaCl, pH 2.8) for 10 minutes. Next the nitrocellulose paper was removed from the glycine buffer and the pH of this selected antibody solution was neutralized by adding 8 mg of Tris per ml of eluate.

2. Electrophoresis and Blotting

Non-denaturing polyacrylamide gel electrophoresis (PAGE) of toxin A was performed in a 5 to 15% gradient slab gel at pH 8.3 (50). Gels were stained with Coomassie Blue R-259. Electrophoretic transfer (Western blotting) of proteins from polyacrylamide gels to nitrocellulose paper was performed as described by Towbin et al. (51).

DNA was separated on a 0.8% agarose gel and stained with ethidium bromide (52). Nick translation with [alpha-^{32}P] dCTP and Southern blot hybridizations were performed as described in the nick translation and sure blot hybridization kits provided by Oncor Inc. (Gaithersburg, Maryland U.S.A.).

3. Cytotoxicity Testing

Supernatant from C. difficile cultures, purified or partially purified toxin A, and crude lysates from E. coli Y1090 (obtained from Promega Biotec) infected with *recombinant lambda* gt11 (52) and induced with isopropyl thio-beta-D galactopyranoside (IPTG) (53), were filter sterilized and cytotoxic activities determined using 3T3 mouse fibroblasts as described previously (35).

4. DNA Sequence Analysis

Sequence analysis of DNA encoding for toxin A was determined using the Sanger chain-termination sequence procedure. Protocols and reagents from the Sequenase™ kit (United States Biochemical Corporation, Cleveland, Ohio) were used for dideoxy sequencing. Nucleotide sequence data was analyzed by an IBM PC-XT computer with programs from International Biotechnologies, Inc., and a Digital Microvax computer using the data base and graphic programs of CageGem.

5. DNA Synthesis

Oligodeoxynucleotide primers were synthesized using beta-cyanoethyl phosphoramidite chemistry in a MilliGen 7500 DNA synthesizer (54). Analysis and purification of oligonucleotides was preformed by either separating DNA by PAGE in a 20% gel, or by fractionating DNA on a trityl-specific reverse phase Delta Pak C18 (Waters Inc.) HPLC column. The three column DNA synthesizer and HPLC systems, which are located in our laboratory, have been

obtained since the funding of the Army contract.

6. Restriction Digestion and Cloning

Restriction endonuclease digestion of DNA, and enzymatic manipulation of DNA for cloning were preformed as described by suppliers. Standard procedures that were used for isolation and manipulation of DNA for cloning are described in several molecular cloning manuals (55, 56).

RESULTS AND DISCUSSION

In the original proposal we reported preliminary screening for toxin A antigen producing recombinants in a lambda gt11 genomic library of C. difficile DNA. Seven positive clones were identified and reported in this proposal; however, in subsequent studies all but one of the identified positive plaques spontaneously lost the C. difficile DNA inserts. This was evident by a reversion from clear to blue plaques, loss of immunological reactivity with toxin A antiserum, and by analyzes of DNA inserts on agarose gels. Lambda cd19 was the only recombinant plaque stable enough to allow for consistent immunological verification; however, this clone reverts from clear to blue plaques at a frequency of 2 percent. (It should be noted that other genes have been screened for in this library and are stable.)

Verification of the immunological identity of the isolated toxin A gene fragment in lambda cd19 was determined by affinity purifying toxin A antibodies specific for lambda cd19 gene product, and using these selected antibodies to probe a Western blot of purified toxin A (fig. 1). E. coli lysates generated from infecting Y1090 with lambda cd19 phage particles and induced with IPTG, were negative for cytotoxic activity on 3T3 mammalian tissue culture cells.

The EcoR1 cloning site in lambda gt11 is contained within a 2.08 kb Sac1-Kpn1 fragment (53); therefore, lambda cd19 DNA was double digested with Sac1-Kpn1. This 2.08 kb Sac1-Kpn1 fragment was increased in size in lambda cd19 to 2.40 kb, thereby demonstrating the presence of a 0.3 kb Taq1 insert (fig. 2). (Note, the genomic library was generated with Taq1.) A 0.3 kb C. difficile DNA insert in lambda cd19 was also demonstrated by hybridizing [alpha-^{32}P] nick translated lambda cd19 DNA to a C. difficile Taq1 chromosomal digest (fig. 3). The [alpha-^{32}P] labeled cd19 probe also hybridized to a 4.5 kb fragment in a Pst1 chromosomal digestion of C. difficile, and a 16 kb fragment in a HindIII digestion (fig. 3).

The toxin A gene fragment in lambda cd19 was not recoverable from EcoR1 digestion of this DNA, even though this insert was ligated into the EcoR1 site of lambda gt11. To determine why this DNA insert did not digest out with EcoR1, the Sac1-Kpn1

fragment was subcloned into M13 (mp18 and mp19) and mapped. Upon simultaneous exposure to EcoR1 and Kpn1 two fragments approximately 1.02 kb and 1.41 kb were produced (fig. 4). The 1.41 kb fragment was generated because an EcoR1 site is located in the multiple cloning region of the M13 vector (this site is contiguous with Sac1). The 1.02 and 1.41 kb fragments were also present with a Sac1-EcoR1-Kpn1 triple digestion of the hybrid DNA. However a double digestion with Sac1-EcoR1 generated a single fragment of 1.41 kb (fig. 4). A restriction map created from these experiments revealed that a single EcoR1 site adjacent to the Sac1 site in lambda cd19 was destroyed or some how protected from digestion following initial cloning (fig. 5).

Attempts were made to sequence the toxin A DNA in the 2.4 kb Kpn1-Sac1 fragment cloned in M13 by using a synthesized 15-mer oligonucleotide which hybridizes to lambda gt11 sequence adjacent to the toxin A insert. A search of GenBank using the IBI software for homology between the primer and M13 suggested that this primer should not bind to M13. However results of sequences generated in these experiments demonstrated that the primer was selectively priming non-targeted sequences. Therefore, the 1.4 kb EcoR1-Sac1 fragment containing the 0.3 kb C. difficile gene was subcloned in M13mp18 and M13mp19 for sequencing. Due to the presence of 1.02 kb of lambda gt11 lacZ DNA preceding C. difficile insert within M13mp18, it was sequenced with a reverse primer, whereas the insert subcloned in M13mp19 was sequenced using M13 universal primer. The DNA sequence and predicted open reading frame of the toxin DNA fragment is shown in figure 6. An hydropathy plot of the open reading frame is given in figure 7, and regions that will be considered in future studies for antigenicity have been indicated.

The 16 kb HindIII fragment identified in the chromosomal digest of C. difficile strain 10463 is being used to clone for the complete toxin A gene or DNA which codes for a complete subunit. DNA surrounding this 16 kb HindIII fragment has been isolated using the Ultrahydrogel DNA HPLC column (Waters Inc.), dephosphorlated, and ligated in the replacement vector lambda 2001. This population of recombinants are being screened with a ^{32}P synthetic DNA probe generated from base pair 38 to 80 (fig. 6). We are currently conduction these experiments. In a recent publication by Wren and coworkers (46) a 14 kb BamH1 fragment was cloned into the EMBL3 replacement vector. This fragment coded for a 235 kDa protein with toxic activity. We will also clone and characterize this fragment.

RECOMMENDATIONS AND CONCLUSIONS

One of the main objectives of this contract is to develop a system for testing epitopes using the expression vector lambda gt11, which includes reporting potential problems and solutions to these problems. From our experience of cloning toxin A and as

well as toxin B gene fragments in lambda gt11, and from personal communications with other investigators, we have identified non-published common problems encountered when using this cloning system. Due to the vector:insert DNA ratio, it is extremely difficult to visualize on an ethidium bromide agarose gel, inserts that are under several hundred base pairs. When foreign DNA inserts are placed in lambda gt11 the EcoR1 insertion sites are often lost. To avoid missing small inserts or clones that have lost the EcoR1 sites, we recommend routinely screening for inserts in lambda gt11 hybrids by double digestion with Sac1-Kpn1 as described in the Results and Discussion.

In conclusion we have cloned, sequenced and partially characterized a toxin A DNA fragment, and initiated work toward cloning the full length toxin gene. During the next year of work we plan to obtain the complete sequence for the toxin gene, predict epitopes, and begin synthesis of these predicted epitopes.

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Figure 1. Purity of toxin A, and confirmation of cloned toxin determinants. PAGE and Western blot analysis were performed as described in materials and methods. Lanes: (1) PAGE of 6 ug purified toxin A. (2) Western blot of toxin A using control toxin A antiserum, 1:200 dilution. (3) Western blot of toxin A with selected antibodies from lambda gt11 control plaques. (4) Western blot of toxin A with antibodies selected from lambda cd19.

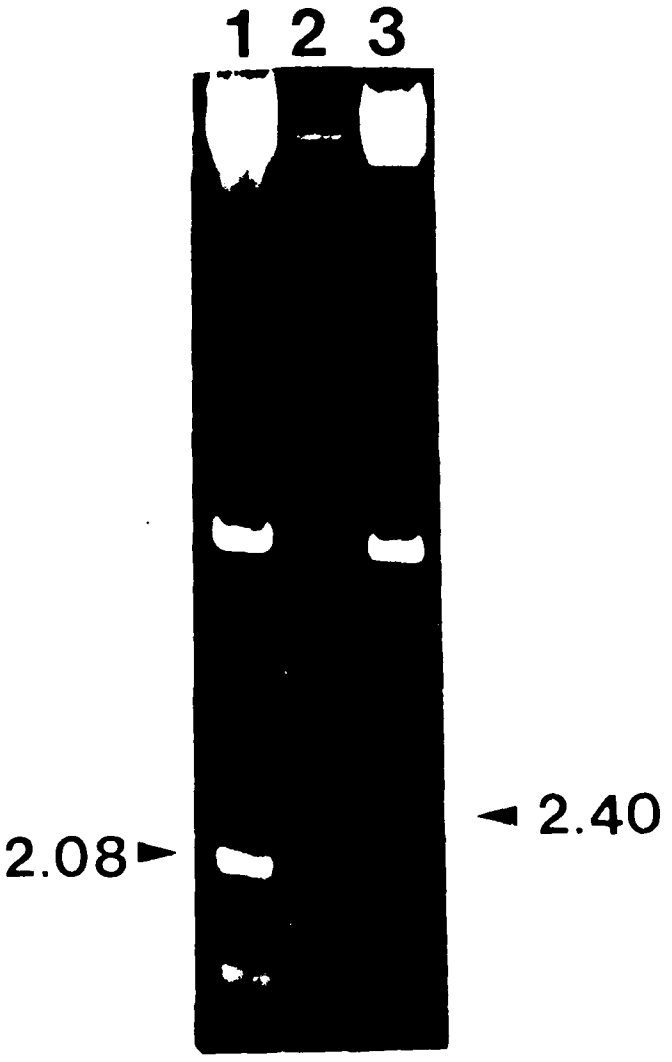

Figure 2. Restriction digestion of lambda gt11 and lambda cd19. Lambda gt11 (lane 1) and lambda cd19 (lane 3) were double digested with Sac1 and Kpn1 as described by supplier. The band immediately above the 2.4 kb fragment in lane 3 represents incomplete digestion of the 2.4 kb fragment and the lower migrating 1.51 kb fragment. Lane 2 contains the molecular weight standard HindIII digested lambda DNA (BRL Inc., Gaithersburg, Maryland USA).

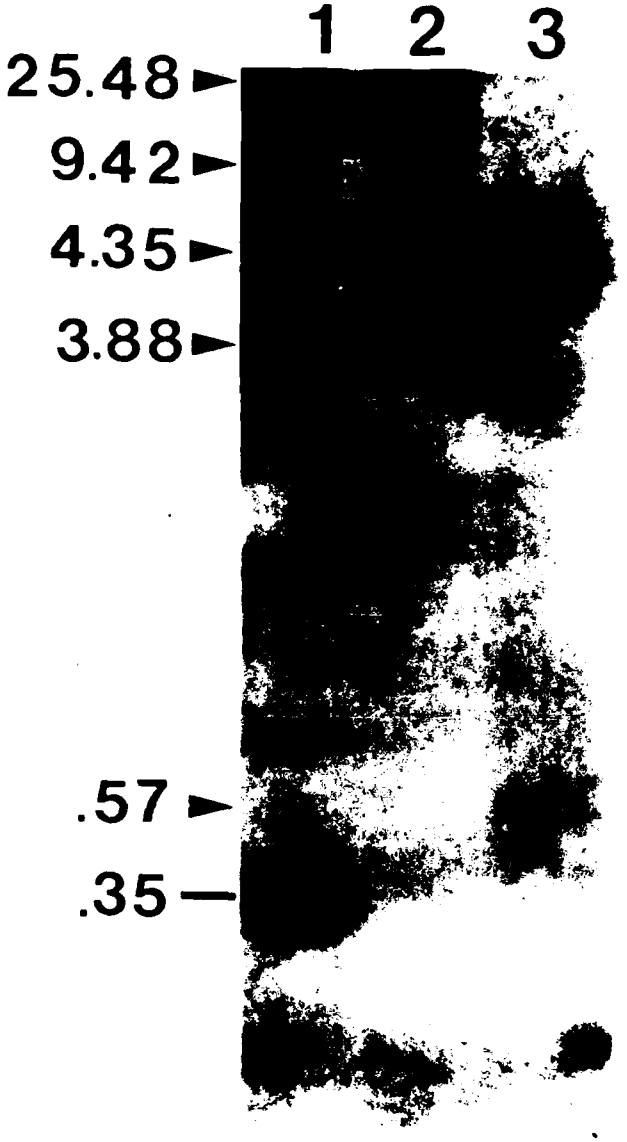

Figure 3. Southern hybridization of restriction digested C. difficile chromosomal DNA with [alpha-^{32}P] dCTP labeled lambda cd19. C. difficile chromosomal DNA was digested with Taq1, lane 1; HindIII, lane 2; and Pst1, lane 3.

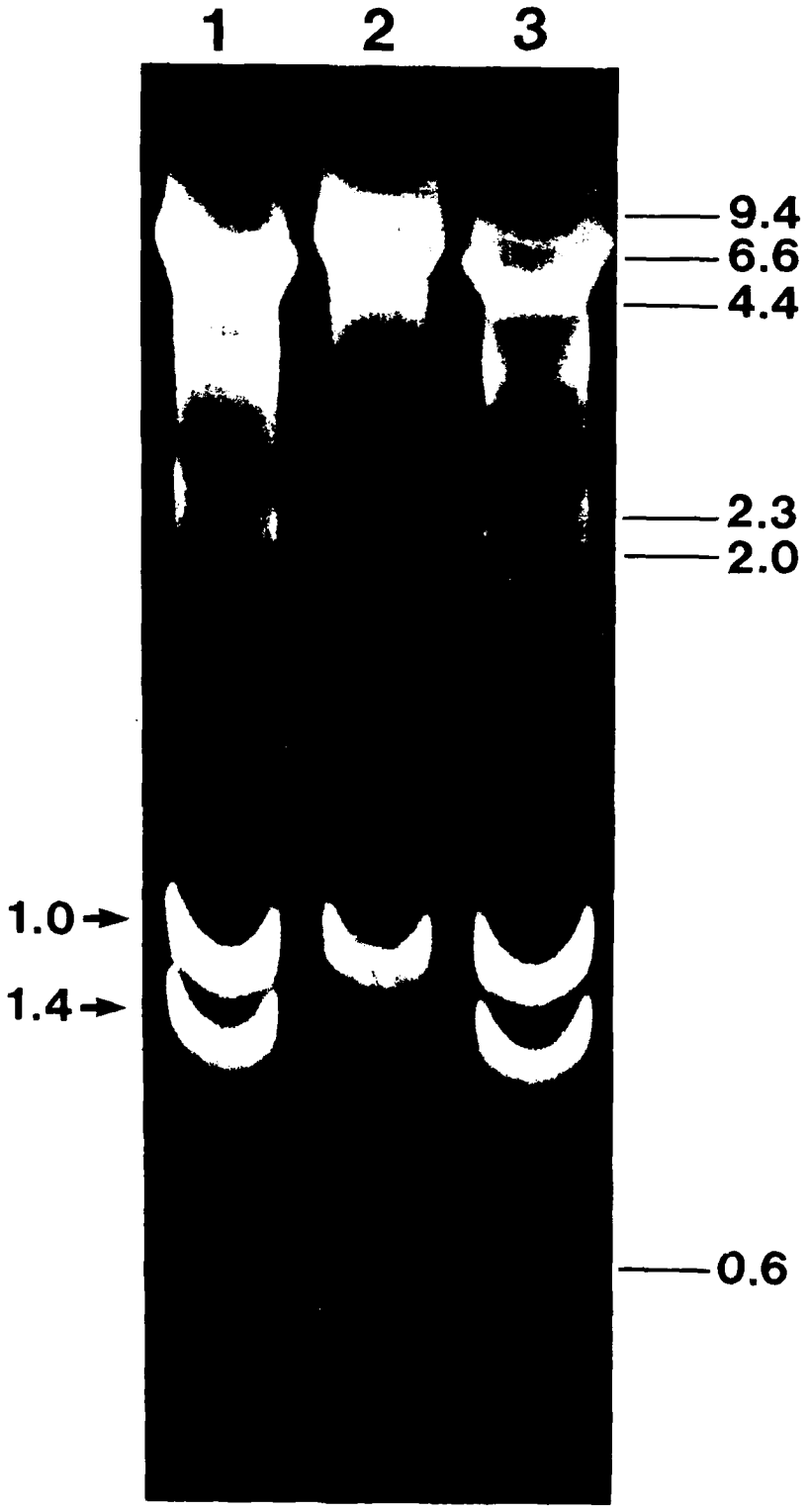

Figure 4. Restriction digestion of cdmp19. Lane 1 was digested with Sac1, EcoR1 and Kpn1; lane 2 with Sac1 and EcoR1; and lane 3 with EcoR1 and Kpn1. A molecular weight marker for the standard HindIII digested lambda DNA is given on the right.

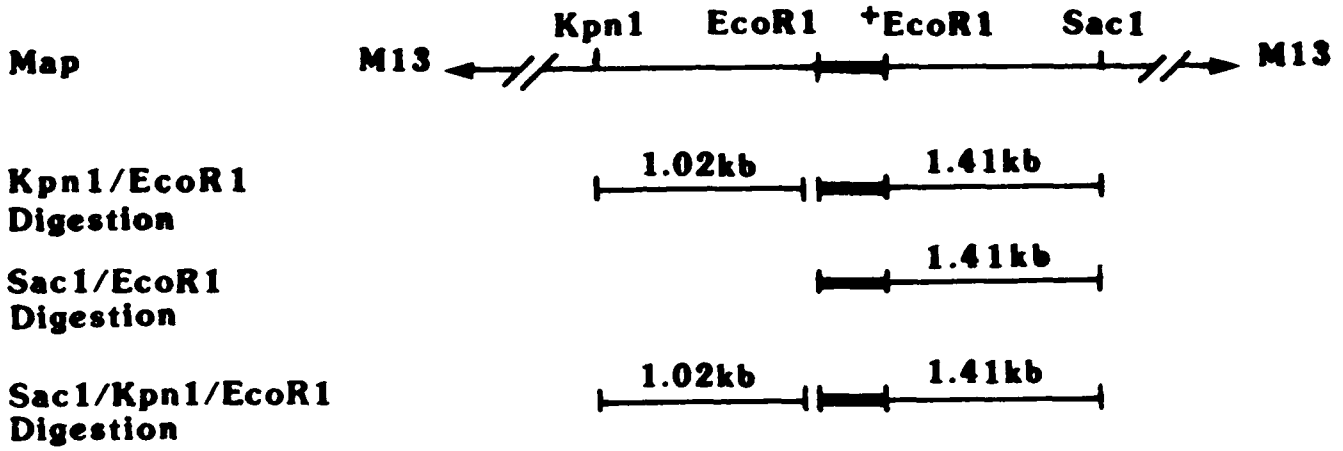

Figure 5. Restriction map of the Kpn1-Sac1 DNA fragment from lambda cd19 that was subcloned into M13. Thick bar represents toxin A DNA, ($^{+}$) designates the damaged EcoR1 site.

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            10          20          30          40          50          60
            *           *           *           *           *           *
CGG GCT CCA GGA GTC GTC GCC ACC AAT CCC CAT ATG GAA ACC GTC GAT ATT CAG CCA TGT
Arg Ala Pro Gly Val Val Ala Thr Asn Pro His Met Glu Thr Val Asp Ile Gln Pro Cys

            70          80          90          100         110         120
            *           *           *           *           *           *
GCC TTC TTC CGC GTG CAG CAG ATG GCG ATG GCT GGT TTC CAT CAG TTG CTG TTG ACT GTA
Ala Phe Phe Arg Val Gln Gln Met Ala Met Ala Gly Phe His Gln Leu Leu Leu Thr Val

           130         140         150         160         170         180
            *           *           *           *           *           *
GCG GCT GAT GTT GAA CTG GAA GTC GCC GCn CCA CTG GTG TGG GCC ATA ATT CAA TTC GCn
Ala Ala Asp Val Glu Leu Glu Val Ala Ala Pro Leu Val Trp Ala Ile Ile Gln Phe Ala

           190         200         210         220         230         240
            *           *           *           *           *           *
CnT CCC GCA GGC CAG ACC GAA AAC GCT CGG GAA GAC GTA CnG GTA TAC ATG TCT GAC AAT
XXX Pro Ala Gly Gln Thr Glu Asn Ala Arg Glu Asp Val XXX Val Tyr Met Ser Asp Asn

           250         260         270         280         290         300
            *           *           *           *           *           *
GGC AGA TCC AGC GTC AAA CAG CGC AGT AAG GCG TCG GAT AGT TTC TTG CGC CTA ATC GAG
Gly Arg Ser Ser Val Lys Gln Arg Ser Lys Ala Ser Asp Ser Phe Leu Arg Leu Ile Glu


CCA GGT TAC
Pro Gly Tyr
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Figure 6. DNA and amino acid sequence of toxin A gene fragment.

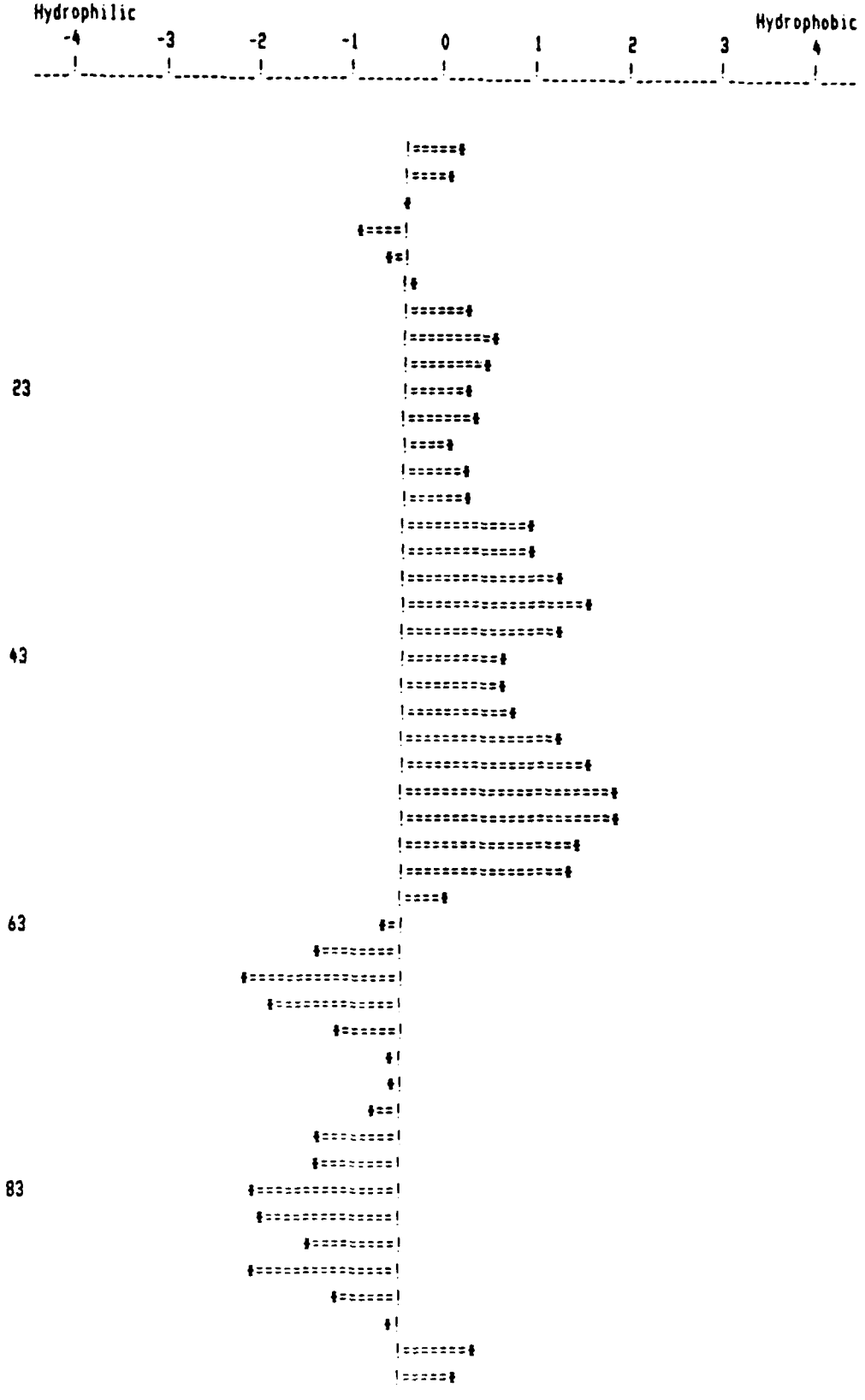

Figure 7. Hydropathy plot of recombinant peptide.